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Clonal Population Structure and Antimicrobial Resistance of *Campylobacter jejuni* in Chicken Meat from Belgium▽

Ihab Habib,[1,4]* William G. Miller,[2] Mieke Uyttendaele,[3] Kurt Houf,[1] and Lieven De Zutter[1]

Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke,[1] and Laboratory of Food Microbiology and Food Preservation, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent,[3] Belgium; Produce Safety and Microbiology Research Unit, USDA, ARS, WRRC, 800 Buchanan Street, Albany, California 94710[2]; and Food Hygiene and Control Division, High Institute of Public Health (HIPH), Alexandria University, 165 El-Horrya Avenue, Alexandria, Egypt[4]

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Campylobacter jejuni* is one of the most important causes of human diarrhea worldwide. In the present work, multilocus sequence typing was used to study the genotypic diversity of 145 *C. jejuni* isolates from 135 chicken meat preparations sampled across Belgium. Isolates were further typed by pulsed-field gel electrophoresis, and their susceptibilities to six antimicrobials were determined. Fifty-seven sequence types (STs) were identified; 26.8% of the total typed isolates were ST-50, ST-45, or ST-257, belonging to clonal complex CC-21, CC-45, or CC-257, respectively. One clonal group comprised 22% (32/145) of all isolates, originating from five different companies and isolated over seven sampling months. Additionally, 53.1% of *C. jejuni* isolates were resistant to ciprofloxacin, and 48.2% were resistant to tetracycline; 28.9% (42/145) of all isolates were resistant to both ciprofloxacin and tetracycline. The correlation between certain *C. jejuni* clonal groups and resistance to ciprofloxacin and tetracycline was notable. *C. jejuni* isolates assigned to CC-21 ($n = 35$) were frequently resistant to ciprofloxacin (65.7%) and tetracycline (40%); however, 90% (18/20) of the isolates assigned to CC-45 were pansusceptible. The present study demonstrates that certain *C. jejuni* genotypes recur frequently in the chicken meat supply. The results of molecular typing, combined with data on sample sources, indicate a possible dissemination of *C. jejuni* clones with high resistance to ciprofloxacin and/or tetracycline. Whether certain clonal groups are common in the environment and repeatedly infect Belgian broiler flocks or whether they have the potential to persist on farms or in slaughterhouses needs further investigation.

Campylobacter jejuni is among the most common bacterial causes of human gastroenteritis worldwide (4, 23). Infected humans exhibit a range of clinical symptoms from mild, watery diarrhea to severe inflammatory diarrhea (14). In addition, *C. jejuni* has been identified as an important infectious trigger for Guillain-Barré syndrome, the most common cause of acute flaccid paralysis in polio-free regions (16). Another issue of concern regarding *Campylobacter* is the increase in antimicrobial resistance appearing in various regions around the world (1). Infection with an antimicrobial-resistant *Campylobacter* strain may lead to a suboptimal outcome of antimicrobial treatment or even to treatment failure (11).

Consumption of contaminated water and raw milk has been implicated in campylobacteriosis outbreaks (23). However, the majority of human cases are sporadic, and consumption or mishandling of contaminated raw or undercooked poultry meat is believed to be an important source of infection. Risk assessment studies, outbreak investigations, and case-control reports all incriminate chicken meat as a major source, perhaps the major source, of food-borne transmission (14, 17, 32, 48). In Belgium in 1999, a controlled withdrawal of poultry products from sale due to alleged dioxin contamination resulted in a 40% reduction in the frequency of human campylobacteriosis (44). Thereafter and since the year 2000, the *Campylobacter* contamination of Belgian poultry carcasses and meat has been monitored by the Federal Agency for the Safety of the Food Chain, and the rate of positive samples is regarded as high. In 2006, 55.5% of cecal samples ($n = 6,443$) from Belgian broilers at slaughter tested positive for *Campylobacter* (3). In 2007, an industry-focused survey reported that 48% of Belgian chicken meat preparations ($n = 656$) were contaminated with *Campylobacter* (19).

Molecular typing is an important tool in elucidating the diversity and transmission routes of *Campylobacter* isolates contaminating the food chain. In the United States, molecular analysis of *Campylobacter* spp. from poultry production and processing environments showed that many of the clones found within a flock are present in the final products, although the diversity of *Campylobacter* isolates in the final product was lower than that observed in the flock (22). Furthermore, numerous molecular epidemiological studies indicate that the genotypes of *C. jejuni* isolated from human cases overlap those of poultry origin (17, 47). Various molecular typing methods for the study of the population structure of *Campylobacter* are currently available (46). Among these, the multilocus sequence typing (MLST) approach is an emerging tool for research on the population structure and molecular epidemiology of *Campylobacter*. The technique is highly reproducible, portable, and easy to interpret, and results can be shared through a publicly accessible online database (31, 34). As such, MLST is becoming an important tool for studying the molecular epide-

* Corresponding author. Mailing address: Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: 32 09 264 73 41. Fax: 32 09 264 74 91. E-mail: ihab.habib@ugent.be.

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miology of *Campylobacter* in a global context. The accumulation of sequence typing data generated from different countries and settings could allow the creation of more-sophisticated models of the epidemiology and evolution of bacterial pathogens and the development of improved approaches for combating their spread (41).

In Belgium, there is a paucity of information regarding the population structure of *Campylobacter* in the chicken meat supply. No population-based surveys have been conducted to investigate the molecular epidemiology of *C. jejuni* in chicken meat at points close to human consumption. In this study, MLST and pulsed-field gel electrophoresis (PFGE) were used to characterize the diversity of, and clonal relationships among, 145 *C. jejuni* isolates from Belgian chicken meat preparations. In addition, we characterized the antimicrobial resistance in this collection and correlated it with *C. jejuni* genotypes.

MATERIALS AND METHODS

Isolate selection and growth conditions. One hundred forty-five *C. jejuni* isolates from 135 chicken meat preparation samples were included in the present study. Isolates were cultured and confirmed to the genus and species levels as previously described (20). The term "chicken meat preparations" refers to portioned, cut, or minced meat, presented as ready-to-cook with added ingredients (e.g., salt, spices, seasoning mix, marinade, or sauce), while the cut surface retains the characteristics of raw meat. Chicken meat samples were collected in a survey in Belgium from February to November 2007 (19). The initial survey targeted 11 companies; however, the *C. jejuni* isolates in the present study come from only five of these companies. The companies selected (coded D, B, C, E, and I) were the top 5 after a descending ranking of all 11 companies according to the incidence of *Campylobacter* spp. in their samples (19). Final packages were sampled either from production lines or from factory chillers before distribution to retail outlets. Four of the five companies own their slaughterhouses, while company B purchases raw meat from cutting plants. Three (B, C, and D) of the five companies are major producers, covering almost 80% of the Belgian market.

The majority of *C. jejuni* isolates (137/145) in this study were cultured by standard direct plating on a selective medium, as described previously (20). The isolate collection was stored at −80°C in sterile full horse blood (E & O Laboratories, Bonnybridge, United Kingdom) and had been minimally subcultured before storage and subsequent testing. When required, isolates were cultured from the frozen stock for 24 h on blood agar plates (Mueller-Hinton agar base CM337 [Oxoid, Basingstoke, United Kingdom] supplemented with 5% [vol/vol] full horse blood [E & O Laboratories]) under a microaerobic atmosphere at 37°C.

DNA extraction and bacterial subtyping by MLST and PFGE. DNA was extracted from an overnight bacterial culture using a commercial DNA extraction kit (AquaPure DNA isolation kit; Bio-Rad) according to manufacturer guidelines. All *C. jejuni* isolates were characterized by MLST on the basis of primers for seven gene targets for each isolate (*aspA* [encoding aspartase A], *glnA* [glutamine synthase], *gltA* [citrate synthase], *glyA* [serine hydroxymethyltransferase], *pgm* [phosphoglucomutase], *tkt* [transketolase], and *uncA* [ATP synthase alpha subunit]), under conditions previously described (10, 31). All allelic sequences were queried against the *C. jejuni* MLST database (http://pubmlst.org/campylobacter/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford. Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the *C. jejuni* MLST database and assigned new numbers.

PFGE was performed using SmaI-digested fragments of bacterial chromosomal DNA as described previously (37). Gel patterns were checked visually and then analyzed using GelCompar software (Applied Maths, Kortrijk, Belgium) with the band tolerance set at 1.5% (34).

Antibiotic susceptibility testing. VetMIC Camp (National Veterinary Institute, Uppsala, Sweden), a MIC-based system, was used for determining antibiotic susceptibility in the isolate collection. The system is commercially available for testing six antimicrobials dried in serial twofold dilutions in 96-well microtiter plates, where each well is inoculated with 100 μl of Mueller-Hinton broth (supplemented with 2.5% lysed horse blood) at an inoculum density of approximately 10^6 CFU/ml. To achieve this inoculum density, bacterial material corresponding to one filled plastic loop (1 μl) was suspended in 2 ml 0.9% saline.

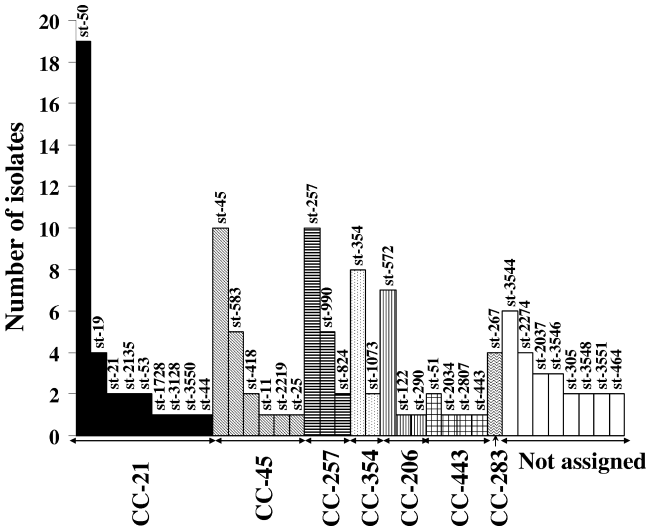

FIG. 1. Distribution of STs and clonal complexes among *C. jejuni* isolates from Belgian chicken meat preparations. Twenty-eight STs contained one isolate each and are not included in the figure.

These bacteria were harvested from a 2-day-old agar culture. This suspension was diluted 1:100 to obtain the final inoculum. The inoculum density was further verified by viable counts after spread plating on blood agar plates. The MIC was read as the lowest concentration completely inhibiting visible growth. Moreover, *C. jejuni* LMG 8841 was included as a quality control strain. The breakpoints of resistance were set as follows: ciprofloxacin, >1 μg/ml; tetracycline, >2 μg/ml; erythromycin, >4 μg/ml; nalidixic acid, >16 μg/ml; gentamicin, >1 μg/ml; streptomycin, >2 μg/ml. These MICs are the breakpoints proposed by the European Food Safety Authority (EFSA) for a harmonized monitoring scheme for antimicrobial resistance in *C. jejuni* isolates from broilers (2).

Characterization of resistance determinants. Total genomic DNA was extracted using a commercial kit (AquaPure DNA isolation kit; Bio-Rad) according to manufacturer guidelines. A mismatch amplification mutation PCR assay for the detection of a mutation (Thr-86 to Ile) in the quinolone resistance-determining region of *gyrA* was performed on all ciprofloxacin-resistant isolates by using primers described by Zirnstein et al. (49). Tetracycline-resistant isolates were screened for *tet*(O) by PCR using primers described by Gibreel et al. (15). PCR screening was also performed on a panel of isolates ($n = 20$) that tested susceptible to ciprofloxacin and tetracycline.

Data analysis. A dendrogram was constructed from the data matrix of MLST allelic profiles with the MEGA (version 3.0) software package (28) by applying the unweighted-pair group method using average linkages. Clonal relationships between the isolates were determined by the eBURST (version 3.0) program with a relaxed group definition of sharing identical alleles at ≥5 of the 7 loci (39). eBURST focuses on those STs that are similar and may share descent from the same founding genotype. Linkage analysis was carried out by calculating the standardized index of association (I_A^S) for the entire *C. jejuni* collection and for selected clonal groups by using the LIAN program, version 3.1 (21). The mean genetic diversity (H) was also estimated using LIAN. If there is a complete linkage equilibrium (an indication of a freely recombining population), the I_A^S is equal to zero, while if there is a complete linkage disequilibrium (an indication of a clonal population structure), then the I_A^S is significantly different ($P < 0.05$) from zero.

RESULTS

Diversity of MLST STs and clonal complexes. Fifty-seven STs were identified among 145 *C. jejuni* isolates from 135 Belgian chicken meat preparation samples. Twenty-eight (49.1%) of the STs were assigned to single isolates, while 27 STs comprised 2 to 19 isolates each. Figure 1 shows that ST-50 was the most frequent (19 of 145 isolates), followed by ST-45 and ST-257, each comprising 10 isolates. Eight, seven, and six

TABLE 1. Characteristics of the novel STs identified in Belgian chicken meat preparations

Sequence type	Clonal complex	No. of isolates	MLST allelic profile[a]						
			aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
ST-3544	NA[b]	6	7	112	5	62	11	3	**249**
ST-3545	CC-1034	1	2	28	4	**329**	74	25	35
ST-3546	NA	3	9	17	5	10	350	**335**	3
ST-3547	CC-206	1	7	84	5	25	2	1	5
ST-3548	NA	2	7	4	2	2	19	1	**248**
ST-3549	CC-702	1	2	52	96	48	127	99	23
ST-3550	CC-21	1	2	1	1	3	258	1	5
ST-3551	NA	2	22	28	4	243	23	29	35

[a] New alleles are given in boldface type.
[b] NA, not assigned.

isolates belonged to ST-354, ST-572, and ST-3544, respectively (Fig. 1). Overall, 8 (14.0%) STs representing 17 (11.7%) isolates had not been reported previously (Table 1); they have been submitted to the *C. jejuni* MLST database (http://pubmlst.org/campylobacter/) and assigned novel ST numbers. The results presented in Table 1 indicate that four of the new STs resulted from new allele sequences, while the remainder represented new combinations of previously described alleles.

The 57 STs were grouped into 16 previously defined clonal complexes; however, 17 STs found in 32 isolates could not yet be grouped into defined clonal complexes (the MLST database was last accessed in January 2009). CC-21 encompassed the largest number of isolates (23.45%), followed by CC-45 (13.79%) and CC-257 (11.72%) (Fig. 1). Thus, together, these clonal complexes encompass almost half (48.96%) of the *C. jejuni* isolates characterized in the present study. Figure 2 shows that isolates assigned to CC-21 and CC-45 were found in meat samples from all five companies. Of note, 70.6% (12/17) of *C. jejuni* isolates assigned to CC-257 and 55% (11/20) of *C. jejuni* isolates assigned to CC-45 were tracked back to samples from company D (Fig. 2).

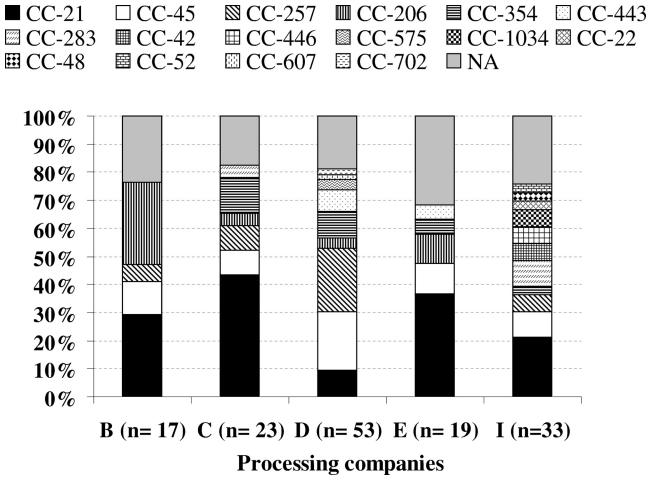

FIG. 2. Distribution of clonal complexes (CC) in relation to the companies from which chicken meat preparations were sampled. Each company is identified by a letter along the *x* axis, with the total number of *C. jejuni* isolates given in parentheses. Each bar represents the proportions of different clonal complexes in the set of isolates from one company. Percentages are given along the *y* axis.

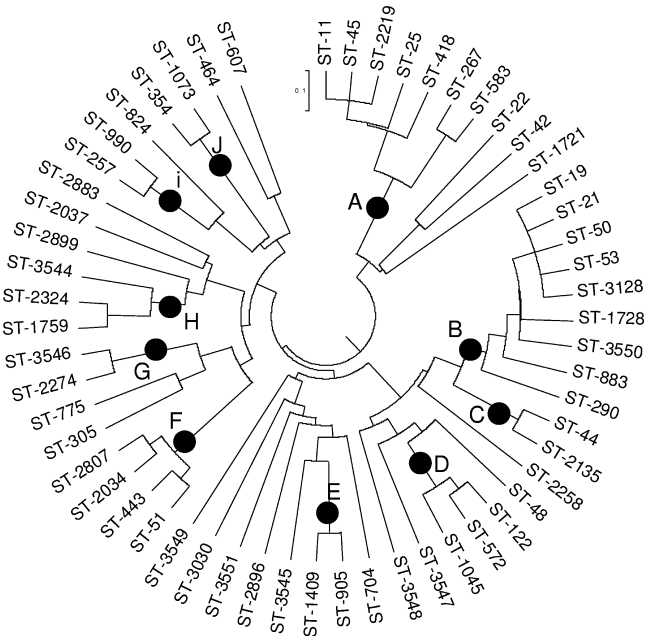

FIG. 3. Phylogenetic relationships among 57 STs characterized for 145 *C. jejuni* isolates from Belgian chicken meat preparations. Letters from A to J indicate eBURST-based clonal groups.

Clonal population structure and recombination analysis. Figure 3 depicts the phylogenetic relationships among the 57 STs identified in the study isolates. The eBURST analysis classified 36 STs into 10 clonal groups (assigned letters from A to J), while the remaining 21 STs were singletons (Fig. 3). "Group" is used below as a neutral term for the collection of STs that are placed together by eBURST according to a definition whereby members of a group share alleles at ≥5 of the 7 loci. The largest clonal group, referred to as group B, comprised 22% of the total *C. jejuni* isolates and contained nine STs (Table 2; Fig. 3), of which ST-50 was the founding genotype (data not shown). To add epidemiological plausibility to the eBURST definition of a clonal group, a correlation between the *C. jejuni* isolates in each group and data on the samples from which they originated is presented in Table 2. This correlation added further insights; for example, the 32 *C. jejuni* isolates in the largest group, clonal group B (in which all isolates belonged to CC-21), were tracked to chicken meat samples from all five companies. Interestingly, these isolates were cultured from a variety of product batches over seven different sampling months (Table 2). On the other hand, the *C. jejuni* isolates in CC-257 ($n = 15$; clonal group I) presumably present a temporal pattern, spanning a period between February and June 2007. Three isolates in this clonal group, from companies C (1-2 and 2-2) and I (7-2), were isolated in February, while the remaining 12 strains (all tracked to company D) were isolated in the subsequent months (Table 2).

On the other hand, the I_A^S of the entire collection ($n = 145$) was 0.4611 ($P < 0.05$), indicating that the alleles were in a linkage disequilibrium (Table 3). In addition, a high degree of genetic diversity ($H = 0.8407$) was evident (Table 3). This finding rejects the null hypothesis of free recombination and indicates that the population has some degree of clonality.

TABLE 2. Characteristics of *C. jejuni* clonal groups as assigned by eBURST analysis[a]

Clonal group	Isolate	Clonal complex	Sequence type	PFGE type	Resistance[b]	Company	Sampling date	Batch[c]
A (n = 24)	422-1	CC-45	ST-11	P50	Pansusceptible	D	22 June 2007	D-30
	501-1	CC-45	ST-2219	P51	Pansusceptible	C	21 Aug. 2007	C-11
	271	CC-45	ST-25	P61	Pansusceptible	D	3 May 2007	D-22
	378	CC-283	ST-267	NT	Pansusceptible	I	12 June 2007	I-14
	379	CC-283	ST-267	NT	Pansusceptible	I	12 June 2007	I-14
	229E1	CC-283	ST-267	NT	Cip + Tet	I	19 Apr. 2007	I-11
	530-3	CC-283	ST-267	NT	Pansusceptible	C	3 Sept. 2007	C-12
	420	CC-45	ST-418	P49	Pansusceptible	D	22 June 2007	D-29b
	421	CC-45	ST-418	P49	Cip	D	22 June 2007	D-30
	268	CC-45	ST-45	P60	Pansusceptible	D	3 May 2007	D-21
	358	CC-45	ST-45	P50	Pansusceptible	D	6 June 2007	D-27
	373	CC-45	ST-45	P49	Pansusceptible	E	8 June 2007	E-6
	432	CC-45	ST-45	P22	Pansusceptible	I	26 June 2007	I-15
	458	CC-45	ST-45	P57	Pansusceptible	E	3 July 2007	E-8
	500	CC-45	ST-45	P23	Cip	C	21 Aug. 2007	C-10
	537	CC-45	ST-45	P52	Pansusceptible	I	10 Sept. 2007	I-17
	122E	CC-45	ST-45	P49	Pansusceptible	I	22 Mar. 2007	I-7
	41-2	CC-45	ST-45	P49	Pansusceptible	B	27 Feb. 2007	B-2
	498-1	CC-45	ST-45	P51	Pansusceptible	B	14 Aug. 2007	B-12
	145	CC-45	ST-583	NT	Pansusceptible	D	28 Mar. 2007	D-9
	91E1	CC-45	ST-583	NT	Pansusceptible	D	14 Mar. 2007	D-7
	89-1	CC-45	ST-583	NT	Pansusceptible	D	14 Mar. 2007	D-5
	90-1	CC-45	ST-583	NT	Pansusceptible	D	14 Mar. 2007	D-6
	95-3	CC-45	ST-583	NT	Pansusceptible	D	14 Mar. 2007	D-8
B (n = 32)	213-1	CC-21	ST-1728	P26	Cip	E	17 Apr. 2007	E-5
	578	CC-21	ST-19	P26	Pansusceptible	B	9 Oct. 2007	B-14
	178E1	CC-21	ST-19	P26	Cip	E	4 Apr. 2007	E-4
	204-1	CC-21	ST-19	P26	Cip	D	12 Apr. 2007	D-18
	530-1	CC-21	ST-19	P26	Cip	C	3 Sept. 2007	C-12
	179-1	CC-21	ST-21	P25	Cip + Tet	E	4 Apr. 2007	E-4
	47-1	CC-21	ST-21	P34	Cip	D	1 Mar. 2007	D-2
	357-2	CC-21	ST-290	P31	Pansusceptible	D	6 June 2007	D-26
	240	CC-21	ST-3128	P27	Pansusceptible	B	24 Apr. 2007	B-6
	187-1	CC-21	ST-3550	P29	Cip	C	11 Apr. 2007	C-4
	109	CC-21	ST-50	P26	Pansusceptible	E	20 Mar. 2007	E-2
	51E1	CC-21	ST-50	P26	Pansusceptible	I	6 Mar. 2007	I-4
	559	CC-21	ST-50	P36	Cip + Tet	D	18 Sept. 2007	D-36
	581	CC-21	ST-50	P36	Pansusceptible	C	9 Oct. 2007	C-13
	629	CC-21	ST-50	P36	Cip + Tet	B	6 Nov. 2007	B-v
	651	CC-21	ST-50	P26	Pansusceptible	D	20 Nov. 2007	D-42
	78E1	CC-21	ST-50	P36	Cip + Tet	C	13 Mar. 2007	C-3
	79E1	CC-21	ST-50	P36	Cip + Tet	C	13 Mar. 2007	C-3
	135-1	CC-21	ST-50	P29	Pansusceptible	B	27 Mar. 2007	B-5
	159-1	CC-21	ST-50	P39	Pansusceptible	I	3 Apr. 2007	I-9
	161-1	CC-21	ST-50	P37	Pansusceptible	I	3 Apr. 2007	I-9
	202-1	CC-21	ST-50	P36	Cip + Tet + Strep + Gent	D	12 Apr. 2007	D-17
	221-1	CC-21	ST-50	P36	Cip + Tet	I	18 Apr. 2007	I-10
	222-1	CC-21	ST-50	P36	Cip + Tet	I	18 Apr. 2007	I-10
	499-1	CC-21	ST-50	P36	Cip + Tet	C	21 Aug. 2007	C-10
	499-3	CC-21	ST-50	P36	Cip + Tet	C	21 Aug. 2007	C-10
	51-1	CC-21	ST-50	P26	Pansusceptible	I	6 Mar. 2007	I-4
	75-1	CC-21	ST-50	P36	Cip + Tet	C	13 Mar. 2007	C-3
	78-2	CC-21	ST-50	P36	Cip + Tet	C	13 Mar. 2007	C-3
	408	CC-21	ST-53	P11	Cip	E	19 June 2007	E-7
	55-1	CC-21	ST-53	P11	Cip	I	6 Mar. 2007	I-4
	501-3	CC-21	ST-883	P32	Cip	C	21 Aug. 2007	C-11
C (n = 3)	177-1	CC-21	ST-2135	P24	Cip + Tet	E	4 Apr. 2007	E-3
	182-1	CC-21	ST-2135	P24	Cip + Tet	E	4 Apr. 2007	E-3
	429-2	CC-21	ST-44	P8	Pansusceptible	B	26 June 2007	B-11
D (n = 9)	557	NA	ST-1045	P42	Pansusceptible	D	18 Sept. 2007	D-34
	442	CC-206	ST-122	P42	Pansusceptible	D	29 June 2007	D-32
	307	CC-206	ST-572	P43	Cip + Tet	B	22 May 2007	B-8
	308	CC-206	ST-572	P43	Cip + Tet	B	22 May 2007	B-9

Continued on following page

TABLE 2—*Continued*

Clonal group	Isolate	Clonal complex	Sequence type	PFGE type	Resistance[b]	Company	Sampling date	Batch[c]
	134-1	CC-206	ST-572	P43	Cip + Tet	B	27 Mar. 2007	B-4
	285-1	CC-206	ST-572	P40	Cip + Tet	E	8 May 2007	E-6
	285-2	CC-206	ST-572	P40	Cip + Tet	E	8 May 2007	E-6
	40-2	CC-206	ST-572	P40	Cip	B	27 Feb. 2007	B-1
	495-3	CC-206	ST-572	P40	Cip + Tet	B	14 Aug. 2007	B-12
E ($n = 2$)	293-1	NA	ST-1409	P56	Cip + Tet	B	11 May 2007	B-7
	461	NA	ST-905	P56	Pansusceptible	E	3 July 2007	E-8
F ($n = 5$)	459	CC-443	ST-2034	P30	Cip	E	3 July 2007	E-8
	556	CC-443	ST-2807	P5	Pansusceptible	D	18 Sept. 2007	D-33
	208-1	CC-443	ST-443	P53	Tet	D	17 Apr. 2007	D-19
	146	CC-443	ST-51	P10	Pansusceptible	D	28 Mar. 2007	D-10
	93-1	CC-443	ST-51	P10	Tet	D	14 Mar. 2007	D-7
G ($n = 6$)	467	NA	ST-2274	P17	Cip + Tet	C	6 July 2007	C-9
	567	NA	ST-2274	P16	Cip + Tet	E	25 Sept. 2007	E-9
	107-1	NA	ST-2274	P16	Cip + Tet	E	20 Mar. 2007	E-1
	296	NA	ST-3546	P7	Cip + Tet	I	11 May 2007	I-12
	531	NA	ST-3546	P7	Cip + Tet	B	3 Sept. 2007	B-13
	214-3	NA	ST-3546	P6	Cip + Tet	E	17 Apr. 2007	E-5
H ($n = 9$)	393-2	NA	ST-1759	P49	Pansusceptible	B	15 June 2007	B-10
	238-2	NA	ST-2274	P18	Tet	C	24 Apr. 2007	C-5
	39-2	NA	ST-2324	P18	Cip + Tet	C	27 Feb. 2007	C-2
	123	NA	ST-3544	P3	Pansusceptible	I	22 Mar. 2007	I-7
	370	NA	ST-3544	P58	Pansusceptible	E	8 June 2007	E-6
	560	NA	ST-3544	P3	Pansusceptible	D	18 Sept. 2007	D-37
	652	NA	ST-3544	P3	Pansusceptible	D	20 Nov-2007	D-43
	654	NA	ST-3544	P3	Pansusceptible	D	20 Nov-2007	D-45
	121-1	NA	ST-3544	P3	Pansusceptible	I	22 Mar. 2007	I-7
I ($n = 15$)	1-2	CC-257	ST-257	P1	Pansusceptible	C	7 Feb. 2007	C-1
	2-2	CC-257	ST-257	P1	Pansusceptible	C	7 Feb. 2007	C-1
	7-2	CC-257	ST-257	P1	Cip + Tet	I	15 Feb. 2007	I-2
	46-4	CC-257	ST-257	P5	Cip + Tet	D	1 Mar. 2007	D-1
	48-1	CC-257	ST-257	P1	Pansusceptible	D	1 Mar. 2007	D-3
	50-2	CC-257	ST-257	P4	Pansusceptible	D	1 Mar. 2007	D-4
	149	CC-257	ST-257	P1	Cip + Tet	D	28 Mar. 2007	D-12
	150-1	CC-257	ST-257	P1	Cip + Tet	D	28 Mar. 2007	D-13
	199-1	CC-257	ST-990	P20	Cip + Tet	D	12 Apr. 2007	D-14
	200-1	CC-257	ST-990	P20	Cip + Tet	D	12 Apr. 2007	D-15
	200-2	CC-257	ST-990	P20	Cip + Tet	D	12 Apr. 2007	D-15
	256	CC-257	ST-990	P20	Cip + Tet	D	27 Apr. 2007	D-19
	272-1	CC-257	ST-257	P19	Pansusceptible	D	3 May 2007	D-22
	272-2	CC-257	ST-257	P1	Tet	D	3 May 2007	D-23
	357-1	CC-257	ST-990	P31	Pansusceptible	D	6 June 2007	D-26
J ($n = 10$)	569	CC-354	ST-1073	P54	Cip	E	25 Sept. 2007	E-9
	116-1	CC-354	ST-1073	P55	Pansusceptible	I	22 Mar. 2007	I-6
	147	CC-354	ST-354	P10	Tet	D	28 Mar. 2007	D-11
	355	CC-354	ST-354	P10	Tet	D	6 June 2007	D-25
	356	CC-354	ST-354	P10	Tet	D	6 June 2007	D-26
	603	CC-354	ST-354	P12	Cip + Tet	D	23 Oct. 2007	D-38
	606	CC-354	ST-354	P12	Cip + Tet	D	23 Oct. 2007	D-41
	236-1	CC-354	ST-354	P13	Cip + Tet	C	24 Apr. 2007	C-5
	239-1	CC-354	ST-354	P6	Cip + Tet	C	24 Apr. 2007	C-6
	37-2	CC-354	ST-354	P13	Cip + Tet	C	27 Feb. 2007	C-2

[a] For each isolate, the results of molecular typing (MLST and PFGE) and antimicrobial resistance are correlated with data tracking the source of the chicken meat. NT, nontypeable (not restricted by the SmaI enzyme); NA, not assigned to a clonal complex.
[b] Cip, ciprofloxacin; Tet, tetracycline; Strep, streptomycin; Gent, gentamicin.
[c] The code for processing batches was identified by the companies.

Moreover, when duplicate STs were removed from the data, the I_A^S dropped significantly, to 0.2280 ($P < 0.05$) (Table 3). The difference in I_A^S between complete and ST-corrected data sets provides a hypothesis of an "epidemic" population structure in which certain clones can arise despite the high degree of genetic diversity. The clonality and the level of recombination differed between *C. jejuni* isolates assigned to CC-21 and CC-45: Table 3 indicates that the rate of recombination events

TABLE 3. I_A^S calculated for the entire *C. jejuni* collection, for STs only, and for selected clonal groups of the collection

Group selection	I_A^S	*P* value[a]		Mean genetic diversity (*H*)
		Parametric	Simulation (1,000 iterations)	
Entire collection (145 isolates)	0.4611	0.0003	0.0003	0.8407 (±0.0069)
STs only (57 STs)	0.2280	0.0008	0.0009	0.8767 (±0.0112)
Clonal group A (7 STs/24 isolates)	0.0901	0.0134	0.0143	0.2350 (±0.0848)
Clonal group B (9 STs/32 isolates)	0.5372	0.0389	0.0421	0.2262 (±0.0677)

[a] The *P* value must be <0.05 for hypothesis rejection. *P* values are derived from the parametric and simulation methods and indicate the significance of linkage disequilibrium.

among group B isolates (assigned to CC-21) was even higher (I_A^S = 0.5372) than that for the whole *C. jejuni* collection. In contrast, a very low I_A^S was estimated for group A (in which most isolates were assigned to CC-45), indicating an extensive level of recombination in strains assigned to this clonal group.

Correlation between PFGE and MLST. Some degree of agreement between MLST and PFGE was evident (Table 2). For example, 12 isolates with the most frequent PFGE type (P36) were all assigned to ST-50. But a one-to-one correlation between PFGE types and STs did not always exist. Some isolates with the same PFGE type had multiple STs, and conversely, isolates with the same ST had multiple PFGE types. For example, seven isolates with P26 had either ST-19, ST-50, or ST-1728 (Table 2). All of these isolates were in clonal group B and were differentiated by changes at only one locus (data not shown). Conversely, isolates with ST-50 (the most frequent ST) had distinct but related (data not shown) PFGE types, assigned as P26, P29, P36, P37, and 39. The overall typeability of isolates by MLST was 100%, and isolates that were not typeable by PFGE were identified by MLST as ST-583 or ST-267 (Table 2). *C. jejuni* isolates assigned to these two STs were cleaved neither by the SmaI nor by the KpnI restriction enzyme despite repeated trials, possibly indicating a site-specific DNA modification, e.g., methylation (18), in ST-583 and ST-267 isolates that interferes with restriction endonuclease action.

Incidence and genetic and genotypic characterization of antimicrobial resistance. Table 4 demonstrates that the *C. jejuni* isolates from Belgian chicken meat showed the highest rates of resistance to fluoroquinolones (nalidixic acid and ciprofloxacin) and to tetracycline. The majority (84.4%) of ciprofloxacin-resistant isolates showed a MIC breakpoint of >8 mg/liter, and the MICs for 87% of tetracycline-resistant isolates were >16 mg/liter. Forty-two isolates (28.9%) showed simultaneous resistance to ciprofloxacin and tetracycline. PCR screening indicated the presence of the characteristic Thr-86-to-Ile mutation in *gyrA* in all ciprofloxacin-resistant isolates (n = 77). In addition, all tetracycline-resistant isolates (n = 70) possessed the *tet*(O) gene.

Figure 4 demonstrates a correlation between certain *C. jejuni* clonal complexes and ciprofloxacin- and tetracycline-resistant isolates. In clonal group B (n = 32), 21 isolates (65.6%) were resistant to ciprofloxacin and 12 isolates (37.5%) were simultaneously resistant to both ciprofloxacin and tetracycline (Table 2). Furthermore, simultaneous resistance to ciprofloxacin and tetracycline was also evident in all isolates in clonal group G (n = 6) and in six of nine isolates in clonal group D (Table 2). All isolates with ST-354 were resistant to tetracycline, representing 80% (8/10) of the isolates assigned to clonal group J (Fig. 4).

On the other hand, 18 of the 20 *C. jejuni* isolates assigned to CC-45 (clonal group A) were susceptible to all six antibiotics included in the study screening panel (Fig. 4).

DISCUSSION

The value of a molecularly based epidemiological study depends largely on the reliability of the typing methods used and on the extent by which isolates in the collection are representative of the population (34, 47). In the present study, a *C. jejuni* collection was selected to represent isolates from five different companies supplying the majority of the Belgian market and also to reflect food processing settings with high *Campylobacter* incidence. The majority of isolates in this collection were cultured by direct plating. Using PFGE typing, Malakauskas et al. (30) reported that direct plating generated *Campylobacter* profiles that were more diverse than those generated by enrichment. Each culture method might have an

TABLE 4. MICs for *C. jejuni* isolates (n = 145) from Belgian chicken meat preparation samples (n = 135)

Antimicrobial	Range of dilutions tested (mg/liter)	Breakpoint for resistance (mg/liter)	No. of isolates with the following MIC (mg/liter):												No. (%) of resistant isolates
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64	
Erythromycin	0.5–64	>4				14	73	52	5	—[a]	—	—	—	1[b]	1 (0.7)
Ciprofloxacin	0.06–8	>1	—	35	22	10	1	1	1	10	65[b]				77 (53.1)
Tetracycline	0.12–16	>2		8	42	16	4	4	2	1	6	61[b]			70 (48.2)
Streptomycin	0.5–64	>2			44[b]	3	72	20	2	1	—	—	1	2[b]	6 (4.1)
Gentamicin	0.12–16	>1	20[b]	1	83	36	3	2	—	—	—				2 (1.4)
Nalidixic acid	1–64	>16					—	2	35	23	5	3	2	75[b]	80 (55.2)

[a] —, no reported results.
[b] MIC above or below the tested range.

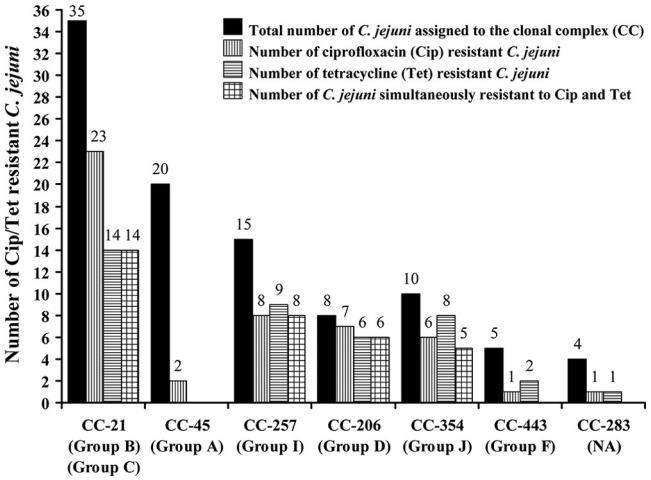

FIG. 4. Distribution of ciprofloxacin- and/or tetracycline-resistant *C. jejuni* isolates from Belgian chicken meat preparations in relation to selected MLST clonal complexes.

effect on the recovery of *Campylobacter* strains (20). Nevertheless, in a previous study (19), we determined that a direct plating method was able to recover more *Campylobacter* isolates from chicken meat preparation samples than enrichment culture. Thereafter, we directed the selection of the typing collection toward isolates generated by direct plating, since they would reflect the majority of the *C. jejuni* population recovered from chicken meat preparations.

In the present study, ST-50, ST-45, and ST-257, assigned to CC-21, CC-45, and CC-257, respectively, were more prevalent in *C. jejuni* isolates from Belgian chicken meat than other STs (Fig. 1). MLST has an established, unified nomenclature that facilitates the integration and comparison of findings from different sources. The frequencies of the main STs and clonal complexes in the present *C. jejuni* collection are comparable to those in other MLST studies, from different European countries, of poultry-related *C. jejuni* isolates (17, 27, 36, 47). However, 14.0% (8/57) of the *C. jejuni* STs identified were novel and have not yet been reported elsewhere (Table 1). We showed in a previous study that a *C. jejuni* strain isolated from a diarrheal sample of a Belgian patient admitted to a hospital in Brussels was also characterized by one of these novel STs, ST-3546 (Table 1) (18a). Between May and September 2007, a preliminary MLST screening of *C. jejuni* isolates ($n = 40$) from Belgian patients with diarrhea (39 cases) indicated that CC-21 was the most frequent clonal complex, identified in 22.5% of the human diarrheal isolates (Habib et al., submitted). In concordance with the previous finding, CC-21 was also more prevalent than other clonal complexes in the present collection of *C. jejuni* isolates recovered from chicken meat in Belgium (Fig. 1; Table 2). The previous findings allow us to hypothesize a possible role for certain *C. jejuni* genotypes in food-borne transmission, with special emphasis on the regional context in Belgium.

The *C. jejuni* isolates in the present study were cultured exclusively from consumer-packed chicken meat preparation samples at the end of processing. The processing of these products involves the exposure of chicken meat to chilling, freezing, and thawing, excessive aeration during mincing, and frequently the addition of marinades, salt, and herbs. The frequent recurrence of certain *C. jejuni* genotypes (Table 2) indicates that these genotypes might have been able to respond better than others to the environmental stresses encountered during the processing of chicken meat preparations. Newell et al. (33) reported that some *C. jejuni* subtypes survived all the processing stages, whereas others survived only to the chilling stages and still others were never recovered from chicken carcasses despite being present in the ceca. In a Danish study (using PFGE, *fla* typing, and serotyping), 7 out of 12 broiler houses were found to carry specific clones over an interval of four broiler flock rotations, or almost 6 months (35). In our study, recurrence of specific *C. jejuni* STs was evident over a period of 7 months, as in *C. jejuni* clonal group B (Table 2). Whether certain clonal groups are so common in the environment that they repeatedly infect Belgian broiler flocks or whether they have the potential to persist on farms or in slaughterhouses needs further investigation.

Almost half of the *C. jejuni* isolates in the present study were resistant to ciprofloxacin, nalidixic acid, and tetracycline. Ciprofloxacin- and tetracycline-resistant isolates showed high MICs (Table 4). Both ciprofloxacin and tetracycline are clinically relevant antimicrobials. Engberg et al. (11) reported that the duration of illness was longer for patients infected with quinolone-resistant *Campylobacter* strains than for those infected with susceptible strains. Previously, a Belgian study reported that 42% of *C. jejuni* isolates from broilers were resistant to ciprofloxacin (42). Taking that finding together with ours, it is critical to accurately measure the prevalence of resistant *Campylobacter* strains and to identify the factors contributing to their high incidence in the Belgian chicken meat supply.

In the present study, clonal groups B, G, and J represent examples of disseminated ciprofloxacin- and/or tetracycline-resistant clones in *C. jejuni* isolates from Belgian chicken meat (Table 2; Fig. 4). Increased resistance might reflect improper use of antimicrobials in broiler farms but might be also due to the origin and transmission of resistance genes and/or the propagation and spreading of resistant clones (5, 24, 25). PCR screening indicated the presence of a mutation in the quinolone resistance-determining region (Thr-86 to Ile) of *gyrA* in all ciprofloxacin-resistant *C. jejuni* isolates characterized in the present study. Luo et al. (29) showed that prolonged experimental colonization of chickens with *C. jejuni* did not result in the reversion or loss of this *gyrA* mutation (Thr-86 to Ile), indicating that this fluoroquinolone resistance-conferring mutation can be stably maintained in the chicken host. The fitness of fluoroquinolone-resistant *C. jejuni* strains was hypothesized in previous studies as well. Humphrey et al. (24) demonstrated that a proportion of ciprofloxacin-resistant *C. jejuni* strains can persist for as long as 4 weeks in commercial broiler flocks after treatment has ceased. Furthermore, Jacobs-Reitsma et al. (25) found that fluoroquinolone-resistant strains emerged in chickens treated with enrofloxacin (but not in chickens treated with flumequine) and persisted for 2 weeks after treatment. On the other hand, *Campylobacter* is known for its capability for natural transformation and conjugation (40, 45). Jeon et al. (26) indicated that natural transformation might not play a significant role in the development of fluoroquinolone-resistant *Campylobacter* strains during the course of fluoroquinolone

treatments. However, they concluded that the horizontal transfer of tetracycline and kanamycin resistance determinants might be mediated by natural transformation. They showed that such natural transformation in *C. jejuni* strains can be abolished by insertional mutagenesis of Cj1211 or the addition of DNase I to the culture medium (26). In our study, *tet*(O) was successfully amplified by PCR screening of all tetracycline-resistant *C. jejuni* isolates. The *tet*(O) gene is widespread in *Campylobacter* strains, and it can be located on either the plasmid or the chromosome (40, 43); however, in the present work we did not analyze its localization. Tetracycline resistance is believed to be typically transferable; the conjugative plasmid carrying *tet*(O) in *C. jejuni* transfers only between *Campylobacter* species (40, 43). Moreover, Avrain et al. (5) showed that there was horizontal transfer of *tet* (O)-positive *Campylobacter* strains between chickens. Thus, the findings of the present study, in concurrence with other reports, corroborate that the retention of ecological fitness in resistant *Campylobacter* strains could create a significant barrier to the elimination of resistant organisms from the chicken meat supply.

The results of the present study highlight an interesting difference between two major MLST complexes, CC-21 and CC-45. Stronger linkage disequilibrium (Table 3) and resistance to ciprofloxacin and tetracycline (Fig. 4) were common features of *C. jejuni* isolates assigned to CC-21. On the other hand, isolates assigned to CC-45 showed a considerable level of recombination (Table 3), and the majority of strains assigned to this clonal complex were pansusceptible to antimicrobials (Fig. 4). Recent research from the United Kingdom hypothesized that CC-45 might be an environmentally adapted complex; this conclusion was supported by a statistical association between *C. jejuni* isolates with CC-45 and the early summer seasonal peak of human cases, rural residence, and a tendency to go fishing before illness (38). Moreover, approximately half of the *Campylobacter* strains recovered from wild bird fecal contamination of playgrounds in parks in a New Zealand city had ST-45 (13). Adding to that, *C. jejuni* isolates from wild geese and European starlings (8, 9), a water puddle outside a broiler chicken house (6), and farm environments (7, 12) have also been characterized as ST-45. In our study, the considerable level of recombination among *C. jejuni* strains assigned to CC-45 could be an indication of processes that might be advantageous for these strains, helping them to adapt rapidly to diverse environmental conditions. Hence, our finding on the extensive level of recombination in CC-45, compared to that in CC-21 and in the entire *C. jejuni* collection in the present study, strengthens the hypothesis on the possible adaptive survival of CC-45 outside the avian host and suggests that environmental exposure might be important for CC-45 contamination in poultry. Whether the differences between CC-21 and CC-45 in recombination patterns and antimicrobial resistance profiles are features of the present isolate collection or general characteristics of the MLST clonal complexes themselves needs to be considered in other investigations.

In conclusion, the present study provides an overview of the clonal population structure of *C. jejuni* isolates from chicken meat at the point closest to human handling and consumption. Despite diversity, clones were revealed in isolates originating from different sources and sampled over a wide temporal span. Furthermore, high resistance to ciprofloxacin and tetracycline was detected in a large number of isolates. The persistence of certain strains in the Belgian chicken meat supply might have an important impact on food-borne transmission to humans. Further investigations are needed to elaborate the mechanisms that allow certain *C. jejuni* genotypes to survive in the chicken meat chain.

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